Journal of Medical Virology 50:82–92 (1996)

Detection of Both Herpes Simplex and Varicella-Zoster Viruses in Cerebrospinal Fluid From Patients With Encephalitis

Inmaculada Casas, Antonio Tenorio, Fernando de Ory, Alvaro Lozano, and José Manuel Echevarría

Department of Diagnosis, Centro Nacional de Microbiología, Virología e Inmunología Sanitarias, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain

Cerebrospinal fluid (CSF) samples from 46 patients with encephalitis were studied for the presence of herpes simplex virus (HSV) types 1 and 2 and/or varicella zoster virus (VZV)-specific DNA sequences by the polymerase chain reaction (PCR) assay. Patients were studied because of detection of intrathecal production of IgG antibody to HSV alone (10 patients, Group A) or to both HSV and VZV (11 patients, Group B) or because of the presence of specific anti-HSV IgG in CSF without evidence of intrathecal antibody production (25 patients, Group C). CSF samples taken between days 1 and 10 from onset of encephalitis were available from all patients, and follow-up samples (taken after 10 days from onset) were obtained from some of them. Positive PCR results were obtained in a total of 13 patients. Four patients (three from Group A and one from Group B) gave amplification of HSV type 1 DNA alone, two patients (both from Group B) showed amplification of VZV DNA alone, and seven patients (all from Group B) gave dual amplification of both HSV type 1 and VZV DNA sequences in CSF. All CSF samples from patients in Group C were negative by PCR. Ten patients with CSF samples positive by PCR lacked a prior history of herpetic cutaneous lesions. In seven patients, serum antibody tests (specific IgM detection and specific IgG avidity assays) identified both primary and recurrent infections. The results suggest that the dual presence of IgG antibody to both HSV and VZV in CSF from patients with encephalitis may reflect in some cases a dual infection of the central nervous system caused by both agents.

KEY WORDS: herpes simplex viruses, varicella-zoster virus, polymerase chain reaction, cerebrospinal fluid, encephalitis

INTRODUCTION

Herpes simplex and varicella-zoster viruses (HSV, VZV) are ubiquitous agents causing a wide range of acute central nervous system (CNS) disease in humans. Encephalitis caused by HSV type 1 (HSV1) or VZV has been described extensively, and most patients with HSV1 encephalitis lack cutaneous vesicles at the onset of neurological disease [Klapper and Cleator, 1992]. This absence of cutaneous lesions also has been documented among patients with VZV-associated acute neurological syndromes [Echevarría et al., 1987, 1994; Mayo and Boos, 1989]. Both primary and recurrent HSV or VZV infections may lead to CNS infection and disease.

Detection of infectious particles in cerebrospinal fluid (CSF) by conventional virus isolation procedures is rarely achieved in patients with neurological disease caused by human alpha-herpesviruses. Demonstration of an intrathecally produced virus-specific antibody response in CSF provides an alternate diagnostic approach in such cases. When tests for HSV IgG and VZV IgG antibody were performed in CSF, the finding of results suggesting intrathecal production of specific antibody to both agents was not unusual [Echevarría et al., 1990b]. Such findings were mainly interpreted as reflecting either cross-reactivity through epitopes common to HSV and VZV antigens or heterotypic responses after polyclonal B-cell activation [Schmidt et al., 1977; Forsgren et al., 1989; Denin and Herb, 1989; Mathiessen et al., 1989; Roberg et al., 1995]. However, dual infections, due to concomitant recurrence of both viruses toward the CNS, cannot be excluded as an explanation [Echevarría et al., 1990a].

In recent years, the polymerase chain reaction (PCR) assay has proven to be a powerful tool for detecting a broad range of infectious agents which can be present

Accepted for publication May 14, 1996.

Address reprint requests to Inmaculada Casas, Service of Diagnostic Microbiology, Centro Nacional de Microbiología, Virología e Inmunología Sanitarias, 28220 Majadahonda, Madrid, Spain.

in extremely small amounts in human tissues or body fluids. Encephalitis due to HSV [Klapper et al., 1990; Aurelius et al., 1991, 1993; Pohl-Koppe et al., 1992; Mertens et al., 1993; Troendle-Atkins et al., 1993; Uren et al., 1993; Guffond et al., 1994] and encephalitis or meningitis due to VZV [Puchhammer-Stöckl et al., 1991; Shoji et al., 1992; Echevarría et al., 1994] have been investigated successfully by testing CSF samples by PCR. Moreover, the PCR assay has been shown to be capable of detecting herpesvirus-specific DNA sequences in other clinical samples that yielded negative results by the current virus isolation procedures [Yoshida et al., 1992; Mertens et al., 1993; Nahass et al., 1995].

In our laboratory, we have been using both virus isolation from CSF and detection of intrathecally produced HSV- and VZV-specific IgG antibody for the diagnosis of acute neurological syndromes of suspected viral origin. In 1991, PCR assays able to detect HSV1, HSV2, and VZV-specific DNA sequences in CSF were introduced as additional diagnostic tools. In this report, we describe the results obtained by PCR in CSF samples from 46 immunocompetent patients with clinical encephalitis who showed the presence of HSV-specific IgG in CSF. Eleven of them were also positive for VZV-specific IgG in CSF, and the correlations made with the virus-specific antibody titers and the albumin concentrations in paired serum–CSF samples suggested that dual intrathecal antibody production to both viruses was produced.

MATERIALS AND METHODS

Patients and Samples

A total of 46 patients with suspected HSV encephalitis were included in the study. All patients presented general features of encephalitis, such as fever, headache, and nuchal rigidity. At least one serum–CSF pair, taken within the first 10 days after onset of neurological symptoms, was available from all patients. Current procedures for isolation of enteroviruses from CSF and detection of IgM antibody to mumps virus in serum and IgG antibody to measles virus in CSF were performed in all cases and gave negative results. Both serum and CSF samples were stored at −20°C until antibody tests and PCR assays were performed.

For inclusion in the study, the presence of specific IgG against HSV in CSF was required. Patients were distributed in three different groups (A, B, and C) following the criteria of intrathecal IgG production against HSV and/or VZV.

Group A consisted of 10 patients with HSV-specific intrathecal IgG antibody production (Table I). Ages ranged from 5 to 75 years. Eight were male and patients 1 and 9 were female. In patients 1, 3, 4, 5, and 10, serum–CSF pair samples taken 14 days after onset of encephalitis were available. A follow-up sample from patient 9 was taken 7 days after onset. In patients 2, 6, and 8, the study was assessed in the serum–CSF pair taken during the acute phase of the illness (days 1, 0, and 5, respectively). Patient 7 presented a recurrent encephalitis with two different episodes: first in November 1989 and second in February 1990.

Group B comprised 11 patients with dual intrathecal IgG production to both HSV and VZV (Tables II and III). Ages ranged from 18 to 75 years (mean 49 years). Ten were male and patient 11 was female. Except in patient 16, follow-up serum–CSF pair samples, taken 10 days after onset, were available. Patients 12 and 20 presented recurrent encephalitis 1 month and 11 months after the initial episode, respectively. Patient 16 had cutaneous herpes zoster at onset of encephalitis, patient 18 showed ophthalmic zoster starting 5 days before onset of encephalitis, and patient 20 had a varicella-like rash at the onset of the first episode of encephalitis. No cutaneous rash or other specific symptoms were observed in the remainder.

Group C comprised 25 patients with no intrathecal production of HSV or VZV IgG. No specific IgG against VZV was detected in CSF samples, and all lacked cutaneous lesions.

Viruses and Control Preparation

Prototype strains of HSV1 (strains HFEM and F), HSV2 (Lovelace), VZV (OKA), human cytomegalovirus (CMV) (AD169), and Epstein-Barr virus (EBV) (P3HR1) were used. All but EBV were propagated in human embryo lung fibroblast (HEF) cells. When cythopathic effect was >75%, the medium was discarded and cells were harvested, aliquoted, and frozen at −20°C. Positive control specimens were serial dilutions of these cell suspensions. A CSF sample from a patient with measles subacute sclerosing panencephalitis lacking detectable levels of antibody against control viruses was used as diluent. This CSF sample and an extract of uninfected HEF cells were used as negative controls.

To check the specificity of the HSV type-specific PCR assay, 10 vesicular fluids from clinical herpetic lesions were processed. Five patients presented oral lesions and the remaining five showed genital lesions. HSV1 or HSV2 were previously isolated from these samples in cell cultures (HEF) and identified by immunofluorescence with type-specific monoclonal antibodies (Syva, Syva Company, Palo Alto, CA, USA). Finally, five CSF samples from patients with acute aseptic meningitis associated to herpes zoster were tested by the HSV type-specific and VZV PCR assays.

Oligonucleotides

Oligonucleotides were designed using computer-assisted analysis of the genomic DNA from sequenced prototype strains of HSV1 (strains Kos, Angeloti, syn17, and SC16), HSV2 (strain 186), and VZV (strain Scott) (EMBL databank, PC-Gene software; IntelliGenetics/Intelli Genetics, Inc. Mountain View, CA, USA).

Specific oligonucleotide sequences for VZV were as previously described [Echevarría et al., 1994]. Specific oligonucleotide sequences for HSV were designed in conserved regions from the DNA polymerase gene, common to both types of HSV. The aim was to amplify both HSV1 and HSV2 DNA sequences in the first amplification reac-

TABLE I. Patients With Single Intrathecal Antibody Production to HSV (Group A)

Patient no.	Age (years)	Sex	Days after onset	HSV serum markers Alb. (mg/dl)	CF	IgG	IgM	HSV CSF markers Alb. (mg/dl)	IgG	PCR[a]	I.alb	Iab.alb
1	67	F	2	nd	<64	6,500	(−)	nd	<20	(+)1	nd	nd
			15	2,650	128	16,000	(−)	10.6	6,000	(+)1	0.004	93.75
2	75	M	1	3,950	64	14,000	(−)	21.5	100	(−)	0.0054	1.31
3	50	M	2	7,100	<64	10,000	(−)	22.8	32	(−)	0.0032	1.00
			20	3,475	<64	12,000	(−)	42.3	36	(−)	0.012	0.25
4	46	M	2	3,692	64	14,000	(−)	44.5	260	(−)	0.012	1.55
			15	6,700	64	12,000	(−)	9.36	30	(−)	0.0014	1.71
5	38	M	7	2,650	<64	18,000	(−)	13.1	80	(−)	0.0049	0.91
			20	2,925	<64	16,000	(−)	12.0	40	(−)	0.0041	0.61
6	5	M	0	4,240	32	14,000	(−)	7.0	40	(−)	0.0017	1.68
7	56	M	0	nd	64	16,000	(−)	—	—	—	nd	nd
			0[b]	3,920	128	45,000	(−)	65.3	4,000	(−)	0.016	5.55
8	75	M	5	5,250	<64	26,000	(−)	250	1,200	(+)1	0.048	0.96
9	6	F	0	2,650	16	12,000	(+)	13.1	80	(+)1	0.0049	1.36
			7	3,109	64	6,500	(+)	12.0	20	(−)	0.0038	0.6
10	10	M	0	2,833	Ac	8,500	(−)	25.7	70	(−)	0.0091	0.9
			120	nd	<64	8,000	nd	—	—	—	nd	nd

[a]HSV type-specific PCR; I.alb, albumin index; Iab.alb, intrathecal antibody production index; CF, complement fixing antibodies; IgG, titer of specific IgG antibody; Ac, anticomplement activity; —, CSF not available; nd, not done; Alb, albumin concentration.
[b]Recurrent encephalitis after 3 months.

tion. Positions of primers were between nucleotides 205 and 753 for HSV1 [Gibbs et al., 1985; Quinn and McGeoch, 1985] and nucleotides 208 and 756 for HSV2 [Tsurumi et al., 1987]. A nested amplification was then carried out using type-specific primers differing in their 3′-end sequences. Primer positions for nested PCR were from 340 to 551 for HSV1 and from 343 to 554 for HSV2. A common probe was used for detection of HSV type-specific amplificates by dot-blot hybridization. Figure 1 shows the HSV and VZV primer sequences and positions.

The degree of homology between the known HSV and VZV DNA polymerase gene sequences was carefully checked in order to exclude cross-ampflication of DNAs. Oligonucleotides were prepared in a Gene Assembler DNA Synthetizer (Pharmacia LKB Biotechnology AB, Uppsala, Sweden).

Viral DNA Amplification

A VZV-specific DNA sequence was amplified by PCR and detected by dot-blot hybridization, as described elsewhere [Echevarría et al., 1994]. HSV1- and HSV2-specific DNA sequences were also detected by dot-blot hybridization after nested PCR.

Viral DNA was extracted from CSF as described previously [Echevarría et al., 1994]. Five microliters of the extract were included in 45 μl of a reaction mixture containing 50 mM Tris-HCl, pH 9.0, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.004% Tween-20, 200 μM of each dNTP, 0.5 μM of the corresponding primers, and 1 unit of Thermostase (Linus DNA Amplification Kit). The mixture was covered with two drops of mineral oil and subjected to an initial PCR cycle (96°C for 1 min, 60°C for 1 min, and 72°C for 30 sec). A further 39 PCR cycles were then undertaken using 96°C and 60°C incubations for 30 sec. A final extension step was carried out at 72°C for 10 min. Nested amplification with internal type-specific primers was done under the same conditions, except for the annealing step (64°C). Amplification was performed in a CC600 Linus Autocycler Plus thermal cycler.

Amplified DNA was applied into a 3% agarose gel (Nusieve-Seakem 3:1; FMC Bioproducts, Rockland, ME) containing 0.5 μg/ml ethidium bromide and visualized after electrophoresis with ultraviolet light. Detection of HSV1-, HSV2-, and VZV-specific amplificates was performed by dot-blot hybridization with ^{32}P-labeled specific probes, as described for VZV DNA detection [Echevarría et al., 1994]. Washing steps for HSV DNA detection were carried out at 52°C, 54°C, and 56°C, respectively.

Antibody Assays

Titration of specific IgG antibody to HSV and VZV in serum and CSF samples and detection of HSV- and VZV-specific IgM in serum were done by indirect enzyme immunoassay (EIA) [Echevarría et al., 1987, 1989]. In samples positive for specific IgM, the avidity of the serum IgG antibody to HSV and VZV was investigated by the urea-reduction assay [Hedman and Seppala, 1988] adapted to a fluoroimmunoassay test [de Ory et al., 1995]. If a reduction higher than or equal to 50% in IgG titer was seen in the sample treated with urea, it was considered positive for low-avidity IgG. Complement-fixing (CF) antibodies to HSV and VZV were measured by a standard micromethod. Specific IgG antibody to measles and mumps viruses in serum and CSF were determined by EIA (Enzygnost; Behring Institute, Marburg, Germany).

The intrathecal synthesis of specific IgG antibody was evaluated by the antibody-albumin index (Iab.alb; ratio of CSF antibody titer/serum antibody titer and albumin CSF/albumin serum). Albumin was measured in serum and CSF by turbidimetry (Turbiquant; Behring Institute). Based on previous experiences, intrathecal anti-

TABLE II. Patients From Group B With Single Positive Result by PCR

Patient no.	Age (years)	Sex	Days from onset	I.alb	HSV						VZV					
					Serum			CSF			Serum			CSF		
					CF	IgG	IgM	IgG	PCR[a]	Iab.alb	CF	IgG	IgM	IgG	PCR	Iab.alb
11	75	F	8	0.0094	<64	12,000	(−)	120	(+)1	1.06	<64	1,000	(−)	<20	(−)	0
			23	0.055	256	125,000	(+)	120,000	(−)	17.5	<64	18,000	(−)	1,600	(−)	1.6
			38	nd	256	120,000	(−)	—	—	nd	<64	6,000	(−)	—	—	nd
			49	nd	256	120,000	(−)	—	—	nd	<64	5,000	(−)	—	—	nd
12	26	M	0	0.0032	256	60,000	(−)	2,000	(−)	10.42	64	1,200	(−)	90	(+)	23.4
			3	nd	256	nd	nd	—	—	nd	64	nd	nd	—	—	nd
			14	nd	256	nd	nd	—	—	nd	128	nd	nd	—	—	nd
			2[b]	nd	256	nd	nd	—	—	nd	64	nd	nd	—	—	nd
			10	nd	256	nd	nd	—	—	nd	64	nd	nd	—	—	nd
			17	nd	256	nd	nd	—	—	nd	64	nd	nd	—	—	nd
			22	0.0038	256	160,000	(−)	2,200	(−)	3.62	64	7,000	(−)	125	(+)	4.69
			24	nd	256	nd	nd	—	—	nd	64	nd	nd	—	—	nd
13	57	M	0	0.0045	64	9,000	(−)	30	(−)	0.74	<64	2,600	(+)	<20	(+)	0
			7	0.0046	64	100,000	(−)	9,000	(−)	19.56	<64	5,500	(−)	5,000	(+)	197.6
			20	0.0069	128	120,000	(−)	50,000	(−)	60.38	<64	1,800	(−)	1,200	(−)	96.6
14	33	M	10	0.0023	<64	550	(−)	70	(−)	55.3	128	34,000	(−)	200	(−)	2.55
			30	nd	128	2,000	nd	—	—	nd	256	30,000	nd	—	—	nd

[a]HSV type-specific PCR; I.alb, albumin index; Iab.alb, intrathecal antibody production index; CF, complement fixing antibodies; IgG, titer of specific IgG antibody; Ac, anticomplement activity; —, CSF not available; nd, not done.
[b]Recurrent encephalitis after 4 weeks.

TABLE III. Patients From Group B With Dual DNA Detection by PCR

Patient no.	Age (years)	Sex	Days from onset	I.alb	HSV						VZV					
					Serum			CSF			Serum			CSF		
					CF	IgG	IgM	IgG	PCR[a]	Iab.alb	CF	IgG	IgM	IgG	PCR	Iab.alb
15	18	M	5	0.004	<8	100	(−)	<20	(+)1	0	<64	90,000	(−)	<20	(−)	0
			14	nd	32	10,000	(+)	—	—	nd	2,048	200,000	(−)	—	—	nd
			24	0.0027	32	10,000	(+)	180	(−)	6.8	2,048	700,000	(−)	6,000	(+)	3.2
16[b]	71	M	10	0.0039	64	30,000	(−)	400	(+)1	3.4	512	25,000	(+)	1,500	(+)	15.4
17	68	M	9	nd	—	—	—	130	(+)1	nd	—	—	—	<20	(−)	nd
			37	0.0066	<64	20,000	(−)	1,200	(−)	10	<64	600	(−)	90	(+)	25
18[c]	30	M	0	0.0073	<64	7,000	(−)	120	(+)1	2.35	1,024	280,000	(+)	2,200	(+)	1.08
			13	0.0287	<64	4,000	(−)	16	(−)	0.14	512	100,000	(+)	20	(+)	0.01
19	44	M	6	0.0065	<64	8,000	(−)	18	(+)1	0.35	<64	3,000	(−)	<20	(+)	0
			14	0.0076	Ac	10,000	(−)	1,900	(−)	26	Ac	5,000	(−)	80	(+)	2.1
20[d]	73	M	2	0.013	<64	10,000	(−)	22	(+)1	0.17	<64	3,600	(−)	36	(+)	0.77
			17	0.014	128	50,000	(−)	6,000	(−)	8.57	<64	4,000	(−)	300	(−)	5.35
			0[e]	0.006	128	8,000	(−)	3,000	(−)	62.5	<64	800	(−)	150	(−)	31.25
21	49	M	2	0.003	<64	18,000	(−)	<20	(+)1	0	<64	240	(+)	<20	(+)	0
			27	0.016	<64	40,000	(−)	200	(−)	0.31	<64	600	(−)	<20	(−)	0
			120	0.007	512	140,000	(−)	20,000	(−)	20.1	<64	9,000	(−)	1,100	(−)	17.2

[a]HSV type-specific PCR; I.alb, albumin index; I.ab.alb, intrathecal antibody production index; CF, complement fixing antibodies; IgG, titer of specific IgG antibody; Ac, anticomplement activity; —, serum or CSF samples not available; nd, not done.
[b]Patient with cutaneous herpes zoster.
[c]Patient with ophthalmic herpes zoster 5 days after onset of encephalitis.
[d]Patient with cutaneous rash similar to varicella.
[e]Recurrent encephalitis after 11 months.

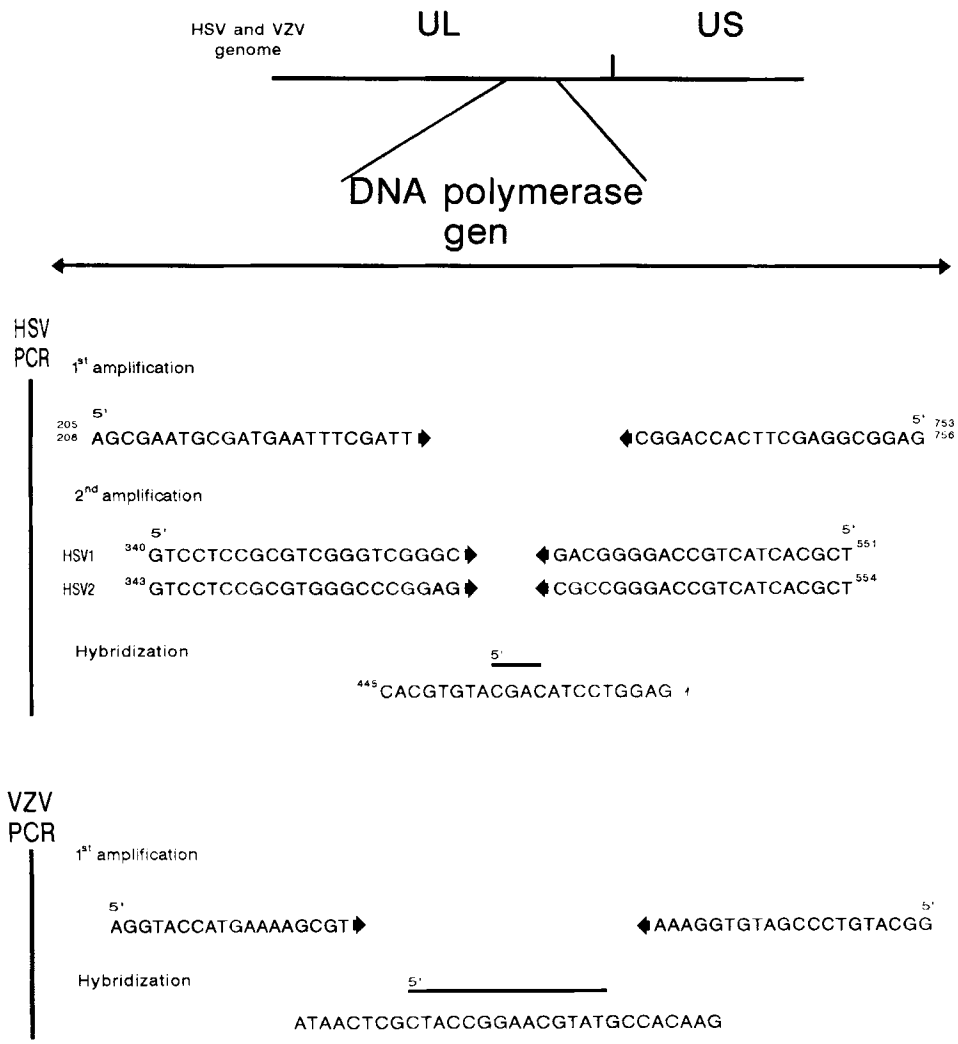

Fig. 1. Primer and probe sequences and relative positions in the DNA polymerase gene used in the first amplification of HSV, nested type-specific HSV, and VZV PCR assays.

body synthesis was considered when the Iab.alb was >0.8 [Arnadottir et al., 1982; Echevarría et al., 1990b].

RESULTS

Analysis of Intrathecal Antibody Production

The values of the Iab.alb in serum–CSF pairs from patients from Groups A and B are shown in Tables I–III. Except in patients 1 and 7, specific HSV Iab.alb values in early samples from Group A ranged between 0.9 and 1.68. Patient 1 showed an increase of antibody titer by complement fixation (CF) assay (serum titer from <64 to 128) and ELISA (CSF titer from <20 to 6,000), and the value of the Iab.alb was 93.75 in a serum–CSF pair taken 15 days after onset. Patient 7 presented, after 3 months, a recurrent encephalitis, the value of the Iab.alb being 5.55.

In Group B, Iab.alb values in early samples ranged between 0 and 10.42 for HSV and 0 and 23.4 for VZV. Increasing values of both HSV and VZV indices were observed in follow-up samples from patients 11, 13, 15, 19, 20, and 21 (Tables II, III).

In Group C, neither HSV- nor VZV-specific intrathecal antibody synthesis was detectable (values <0.8; data not shown).

Validation of PCR Assays

The specificity of the PCR assays is assessed in Figure 2. No hybridization signals were obtained for heterologous viral amplificates or products obtained from the amplification of the CSF used as diluent (Fig. 2A). Signals were specific for the virus and the set of primers used. Vesicular fluids from clinical herpetic lesions identified the HSV type previously identified by immunofluorescence (Fig. 2B). Fluids from oral herpetic lesions 1 to 5 were positive for HSV1 and from genital lesions 6 to 10 for HSV2. Results obtained with CSF samples from patients who presented aseptic meningitis associated to cutaneous herpes zoster are also shown in Figure 2 (B,

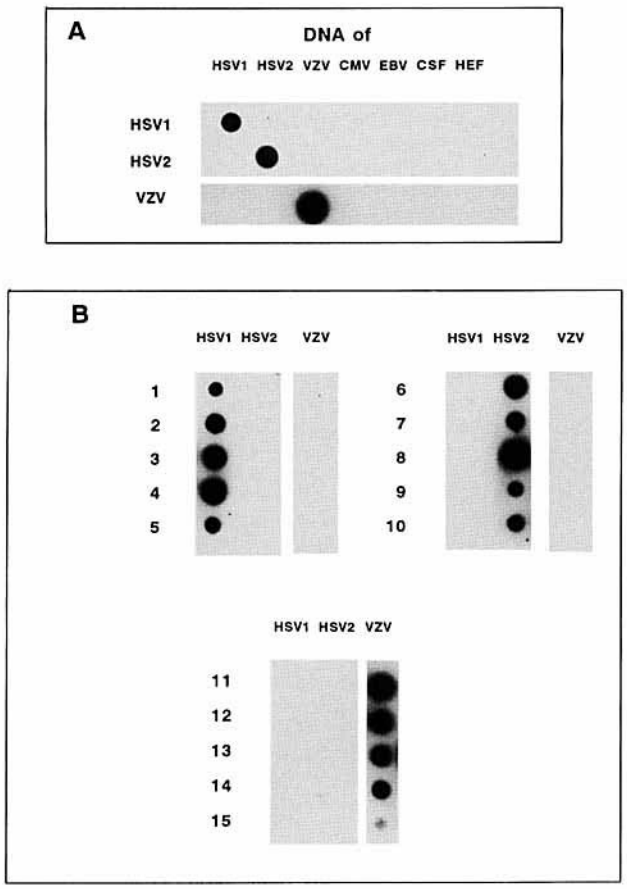

Fig. 2. **A:** Dot-blot hybridization analysis of amplified products obtained with the HSV1, HSV2, and VZV PCR assays on extracts of HEF-uninfected cells and HEF cells infected with homologous and heterologous human herpesviruses. **B:** Dot-blot hybridization analysis of amplified products obtained after type-specific PCR assay from 10 vesicular fluid samples taken from herpetic lesions produced by either HSV1 (1–5) or HSV2 (6–10) and specific VZV PCR assay from five CSF samples obtained from five patients with acute aseptic meningitis associated to cutaneous herpes zoster (11–15).

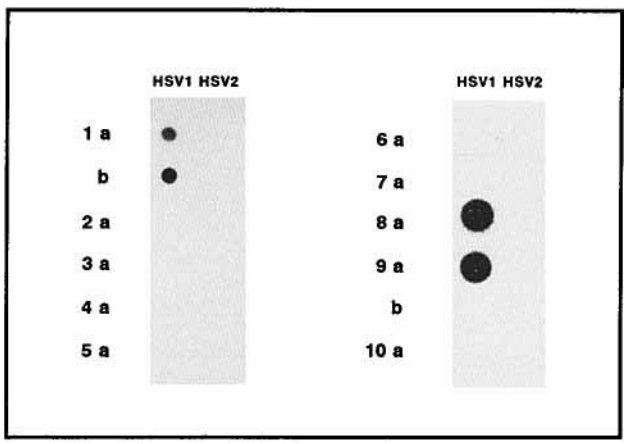

Fig. 3. Detection of HSV1 and HSV2 sequences in early (a) and follow-up (b) CSF samples from 10 patients from Group A who showed intrathecal synthesis of IgG antibody to HSV alone in CSF samples.

11–15). Positive signals confirmed prior results [Echevarría et al., 1994]. No hybridization signals were obtained with these CSF samples and the HSV-specific set of primers. No cross-reactions between the PCR assays were seen.

Amplification of Viral DNA in CSF Samples

CSF samples from 46 patients were studied by PCR. Figures 3 and 4 show the PCR results obtained in samples from patients from Groups A and B. In Group A, HSV1 DNA was detected in CSF samples from patients 1, 8, and 9. Patient 1 was positive for HSV1 in both early and late CSF samples (2 and 15 days after onset, respectively). Negative results were obtained in patients 2–7 and 10 (Fig. 3). Among patients with Group B, specific amplification of HSV1 and/or VZV DNA sequences was obtained in 10 cases. The exception was patient 14, whose first CSF sample was taken 10 days after onset. Single positive results were obtained in three cases: one patient with HSV1 (patient 11) and two patients with VZV (patients 12 and 13) (Table II). Dual amplification of HSV1 and VZV DNAs was found in seven cases (Table III). The simultaneous presence of both viral DNAs was seen in early CSF samples from five patients (16, 18, 19, 20, and 21), two of whom (patients 18 and 19) showed persistence of VZV DNA in follow-up CSFs taken 13 and 14 days from onset, respectively. In patients 15 and 17, HSV1 DNA was initially detected in early CSF samples taken 5 and 9 days after onset, being then cleared from follow-up CSFs where the VZV DNA rose (Fig. 4; Table III). Follow-up samples were available in 10 patients of Group B and six were positive for VZV DNA alone (patients 12, 13, 15, 17, 18, and 19). Four of them showed dual amplification, either simultaneous or sequential (patients 15, 17, 18, and 19). Tables I–III summarize all results obtained by PCR on CSF samples from patients from Groups A and B. No HSV DNA was detected in samples from patients from Group C (data not shown).

Serum Antibody Responses

Table IV lists the samples from patients positive for HSV- or VZV-specific IgM in serum and gives the results obtained by the IgG-avidity assays. Primary infections, as indicated by the presence of low-avidity IgG antibody, were diagnosed in patients 9 and 15 (HSV) and patients 16 and 18 (VZV). In the remaining five cases, serum markers were suggestive of recurrent infections. All patients were positive by PCR in CSF for either a single virus or both.

DISCUSSION

The finding of viral particles, infectious virus, or virus-infected cells in CSF is taken as evidence of viral infection of the CNS and used to establish a viral etiology for neurological diseases [Grandien and Olding-Stenkvist, 1984]. For viruses such as the human alpha-herpesviruses, which are able to establish latency in the nervous tissues, recurrence may coincide with unrelated clinical diseases [Gilden et al., 1992]. Therefore, the etiological link between herpesviruses and neurological disease is

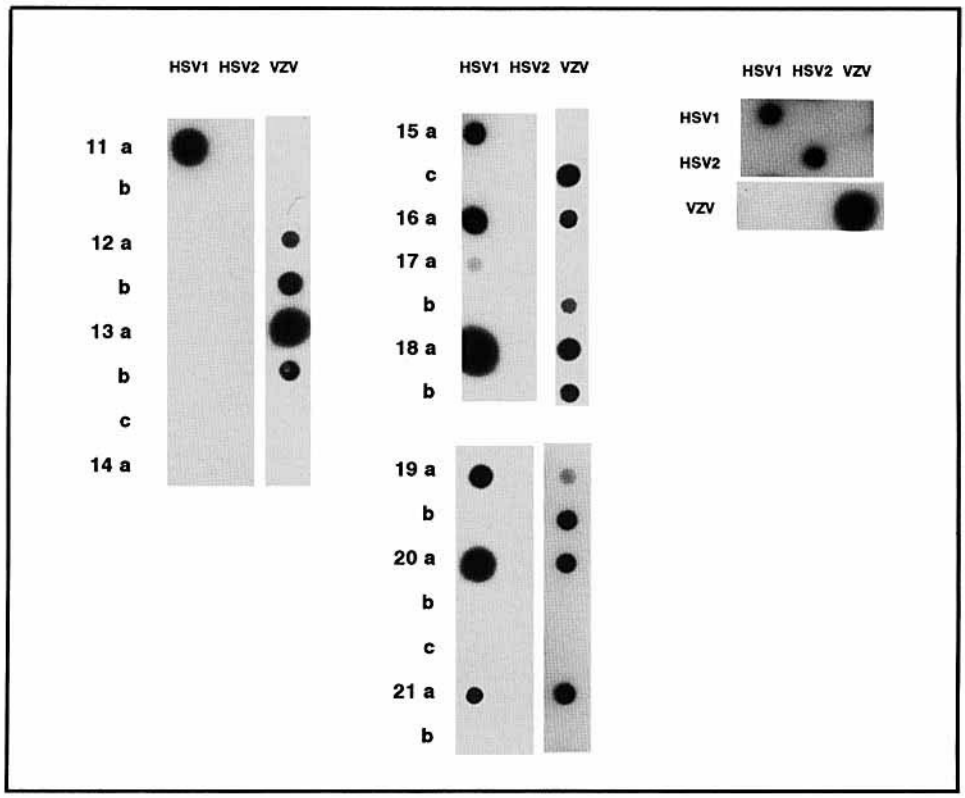

Fig. 4. Detection of HSV1, HSV2, and VZV DNA sequences in early (a) and follow-up (b,c) CSF samples from 11 patients from Group B who showed intrathecal synthesis of IgG antibody to both HSV and VZV in CSF samples.

likely to require additional evidence, such as a significant serum antibody response or a specific intrathecal antibody production.

Since isolation of virus from CSF is a very uncommon finding in patients with HSV and VZV encephalitis, PCR assays for detecting HSV and VZV DNA have been developed and have proven to be a rapid and useful diagnostic alternative to cell culture [Klapper et al., 1990; Puchhammer-Stöckl et al., 1991; Furuta et al., 1992; Aurelius et al., 1993; Mertens et al., 1993; Uren et al., 1993; Troendle-Atkins et al., 1993; Echevarría et al., 1994; Guffond et al., 1994]. In this study, we have been able to demonstrate, using HSV- and VZV-specific PCR assays, the presence of both HSV and VZV DNAs in CSF samples from seven patients with acute encephalitis, thus suggesting that both agents were etiologically involved. Furthermore, additional evidences supporting such involvement include: (1) the presence of specific IgG antibody to both viruses in CSF samples, (2) data suggesting the intrathecal origin of these dual antibody responses, (3) the finding of significant dual serum antibody response in some cases, and (4) the exclusion of other viral agents that may produce neurological syndromes (i.e., enteroviruses, measles, and mumps viruses). However, all these facts would be significant only if both PCR assays and antibody tests performed in the CSFs were really giving specific results.

Several experiments have been done in this study to assess the specificity of the PCR assays used. These included the cross-testing of high titered prototype strains of HSV1 (HFEM, strains F and syn17), HSV2 (Lovelace), VZV (OKA), CMV (AD169), and EBV; human DNA from HEF cell cultures; and 15 wild strains of HSV1, HSV2, and VZV (five strains each) isolated in our laboratory from Spanish patients. Amplification of heterologous DNA was never obtained, whereas amplification of homologous DNA was seen in all cases. Alignment of the HSV and VZV sequences entrusted to the EMBL databank showed high homology between HSV type 1 and HSV type 2 DNA polymerase gene sequences. For HSV1 and HSV2 typing, oligonucleotides were designed with at least three mismatches in the 3′ extreme of each specific primer. To exclude cross-amplification, homology between HSV and VZV sequences in the DNA polymerase gene has been carefully revised for primer design.

It is well known that HSV and VZV share antigenic epitopes that may produce cross-reactivity in these tests [Forsgren et al., 1989]. In fact, such cross-reactivity has been used previously to explain the finding of dual antibody responses to HSV and VZV in CSF samples from patients with neurological diseases [Denin and Herb, 1989; Mathiessen et al., 1989; Roberg et al., 1995]. However, PCR studies in patients with such responses have

TABLE IV. Specific IgG Antibody Avidity in Patients With IgM Response in Serum Samples

Patient no.	Age (years)	Days from onset	HSV serum markers					VZV serum markers				
			CF	IgM	IgG	% Reduction ab.avidity	CSF PCR[a]	CF	IgM	IgG	% Reduction ab.avidity	CSF PCR
9	6	0	16	(+)	12,000	67% Low	(+)1	1,024	(−)	120,000	1% High	(−)
		7	64	(+)	6,500	68% Low	(−)	512	(−)	90,000	11% High	(−)
11	75	8	<64	(−)	12,000	23% High	(+)1	<64	(−)	1,000	45% High	(−)
		23	256	(+)	125,000	nd	(−)	<64	(−)	18,000	nd	(−)
		40	256	(−)	120,000	nd	—	<64	nd	6,000	nd	—
		50	256	(−)	120,000	nd	—	<64	nd	3,000	nd	—
13	57	0	64	(−)	9,000	21% High	(−)	<64	(+)	2,600	22% High	(+)
		7	64	(−)	100,000	nd	(−)	<64	(−)	5,500	nd	(+)
		20	128	(−)	120,000	nd	(−)	<64	(−)	1,800	nd	(−)
15	18	5	<8	(−)	100	(−)[b]	(+)1	<64	(−)	90,000	nd	(−)
		14	32	(+)	10,000	76% Low	—	2,048	(−)	200,000	2% High	—
		24	32	(+)	10,000	nd	(−)	2,048	(−)	700,000	nd	(+)
16	71	10	64	(−)	30,000	9% High	(+)1	512	(+)	25,000	52% Low	(+)
18	30	0	<64	(−)	7,000	12% High	(+)1	1,024	(+)	280,000	70% Low	(+)
		13	<64	(−)	4,000	nd	(−)	512	(+)	100,000	nd	(+)
21	49	2	<64	(−)	18,000	20% High	(+)1	<64	(+)	240	20% High	(+)
		27	<64	(−)	40,000	nd	(−)	<64	(−)	600	nd	(−)
		120	512	(−)	140,000	nd	(−)	<64	(−)	9,000	nd	(−)

[a]HSV type-specific PCR; CF, complement fixing antibodies; —, CSF sample not available; nd, not done.
[b]Sample negative by fluoroimmunoassay test.

not been reported yet. In our study, three patients with encephalitis who presented a dual intrathecal IgG antibody response to both HSV and VZV yielded positive PCR results in CSF for one virus alone (HSV in patient 11 and VZV in patients 12 and 13). Therefore, our results suggest that these cases are likely to respond to cross-reactivity by the antibody tests. It is interesting to note that in these patients the Iab.alb showed higher values for the virus that was detected by PCR than for the heterologous virus, thus confirming our previous data regarding the use of these values to identify the causal agent in such cases [Echevarría et al., 1990a]. In contrast, dual amplification of both HSV and VZV DNA-specific sequences was achieved in CSF samples from seven patients (Table III). Moreover, two of them (patients 15 and 21) showed a dual serum antibody response detected by CF, EIA, or both. We have not done experiments to specifically exclude a cross-reactivity in the antibody tests in these cases. However, the cross-reactivity in both IgG antibody assays and PCR tests in the CSF samples of these seven patients seems an unlikely explanation for the findings.

On the basis of prior experience [Echevarría et al., 1987, 1990b], we used in this study a low cut-off value (0.8) to evaluate the significance of the Iab.alb in regard to the intrathecal antibody synthesis. However, this criterion has influenced the interpretation of the antibody tests in only one patient with dual amplification of HSV and VZV DNA in CSF samples (patient 18). In the remaining six patients, the Iab.alb ranged between 2.1 and 62.5, thus exceeding the criteria established by other authors (1.4–1.9) [Klapper et al., 1981, 1990]. Moreover, the results obtained in other patients with single amplification of HSV DNA in CSF (patients 8, 9, and 11) show that a higher cut-off value would have decreased sensitivity in the early stages of the disease.

Specific IgM is usually taken as a marker of acute, primary viral infection. Herpesvirus recrudescences may induce specific IgM responses, and the IgG avidity test has been shown to be able to differentiate these cases from those responding to acute primary infections [de Ory et al., 1995]. In our study, seven patients showed an IgM antibody response to either HSV or VZV at the time of detection of viral DNA sequences in CSF. Most of them presented high-avidity IgG antibody from the early stages of the disease, thus confirming that HSV and VZV encephalitis are often associated with recrudescences. However, IgG avidity patterns suggesting a primary anti-VZV response were seen in two of the three patients with dual detection of HSV and VZV in CSF and anti-VZV IgM in serum.

In conclusion, it seems likely from these results that dual infections of the CNS caused by HSV and VZV might exist and would explain some cases of CNS disease showing dual intrathecal antibody production to both viruses. Both primary and recurrent infections seem to be involved in such cases, but dual recurrences seem to be more frequent and can be found either in the presence or the absence of a significant serum antibody response. However, some concern still remains regarding the specificity of the antibody assays and the PCR tests, so a further confirmation of the results obtained would be required. Testing of CSF samples by HSV- and VZV-specific PCR assays using primers designed in different genomic regions would exclude cross-reactivity by unusual viral strains or contamination of samples by amplified products. Moreover, testing for HSV- and VZV-specific antibody in CSF using selected, virus-specific glycoproteins known to exclude cross-reactivity would help the interpretation of the antibody assays. Both studies are under current investigation.

ACKNOWLEDGMENTS

This work was supported by a grant from the Instituto de Salud Carlos III, Spanish Ministry of Health (I.C.). Authors are members of the European Union Concerted Action on Virus Meningitis and Encephalitis.

REFERENCES

Arnadottir T, Reunanen M, Salmi A (1982): Intrathecal synthesis of virus antibodies in multiple sclerosis patients. Infection and Immunology 38:399–407.

Aurelius E, Johansson B, Sköldenberg B, Staaland A, Forsgren M (1991): Rapid diagnosis of herpes simplex encephalitis by nested polymerase chain reaction assay of cerebrospinal fluid. Lancet 337:189–192.

Aurelius E, Johansson B, Sköldenberg B, Forsgren M (1993): Encephalitis in immunocompetent patients due to herpes simplex virus type 1 or 2 as determined by type-specific polymerase chain reaction and antibody assays of cerebrospinal fluid. Journal of Medical Virology 39:179–186.

Denin RH, Herb E (1989): Immunological diagnosis in viral infections of the central nervous system: Course of antibody titers against homo- and heterologous viruses. Medical Microbiology and Immunology 178:255–258.

de Ory F, Casas I, Domingo CJ, Echevarría JM (1995): Application of fluoroimmunoassay to the identification of low avidity specific IgG against pathogenic human viruses and *Toxoplasma gondii*. Clinical and Diagnostic Virology 3:323–332.

Echevarría JM, Martínez-Martín P, Téllez A, de Ory F, Rapún JL, Bernal A, Estévez E, Nájera R (1987): Aseptic meningitis due to varicella-zoster virus: Serum antibody levels and local synthesis of specific IgG, IgM and IgA. Journal of Infectious Diseases 155: 959–967.

Echevarría JM, de Ory F, León P, Téllez A (1989): Definition of high-proficiency serological markers for diagnosis of varicella-zoster virus infections by enzyme-immunoassay. Journal of Medical Virology 27:224–230.

Echevarría JM, Téllez A, Martínez-Martín P (1990a): Subclass distribution of the serum and intrathecal IgG antibody response in varicella-zoster virus infections. Journal of Infectious Diseases 162:621–626.

Echevarría JM, de Ory F, León P, Téllez A, Martínez-Martín P, Martín-Fontelos P (1990b) Comparative sensitivity of different criteria for interpreting viral antibody tests in cerebrospinal fluid from patients with acute viral meningitis. Serodiagnosis and Immunotherapy in Infectious Disease 4:193–199.

Echevarría JM, Casas I, Tenorio A, de Ory F, Martínez-Martín P (1994): Detection of varicella-zoster virus-specific DNA sequences in cerebrospinal fluid from patients with acute aseptic meningitis and no cutaneous lesions. Journal of Medical Virology 43:331–335.

Forsgren M, Sköldenberg B, Jeansson S, Grandien M, Blomberg J, Juto P, Berström T, Olding-Stenkvist E (1989): Serodiagnosis of herpes encephalitis by indirect enzyme-linked immunosorbent assay: Experience from Swedish antiviral trial. Serodiagnosis and Immunotherapy Infectious Diseases 3:259–271.

Furuta Y, Takasu T, Fukuda S, Sato-Matsumura K, Inuyama Y, Hondo R, Nagashima K (1992): Detection of Varicella-Zoster virus DNA in human geniculate ganglia by polymerase chain reaction. Journal of Infectious Diseases 166:1157–1159.

Gibbs JS, Chiou HC, Hall JD, Mount DW, Retondo MJ, Weller SAK, Coen DM (1985): Sequence and mapping analysis of the herpes

simplex virus DNA polymerase gene predict a C-terminal substrate-binding domain. Proceedings of the National Academy of Sciences USA 82:7969–7973.

Gilden DH, Dueland AN, Devlin ME, Mahalingam R, Cohrs R (1992): Varicella-zoster virus reactivation without rash. Journal of Infectious Diseases 166(suppl 1):30–34.

Grandien M, Olding-Stenkvist E (1984): Rapid diagnosis of viral infections in the central nervous system. Scandinavian Journal of Infectious Diseases 16:1–8.

Guffond T, Dewilde A, Lobert PE, Caparros-Lefebvre D, Hober D, Wattre P (1994): Significance and clinical relevance of the detection of herpes simplex virus DNA by the polymerase chain reaction in cerebrospinal fluid from patients with presumed encephalitis. Clinical Infectious Diseases 18:744–749.

Hedman K, Seppala I (1988): Recent rubella virus infection indicated by a low avidity of specific IgG. Journal of Clinical Immunology 8:214–220.

Klapper PE, Cleator GM (1992): The diagnosis of herpes simplex encephalitis. Reviews in Medical Microbiology 3:151–158.

Klapper PE, Cleator GM, Logson M (1981): Rapid non-invasive diagnosis of herpes encephalitis. Lancet 2:607–609.

Klapper PE, Cleator GM, Dennett C, Lewis AG (1990): Diagnosis of herpes encephalitis via Southern blotting of cerebrospinal fluid DNA amplified by polymerase chain reaction. Journal of Medical Virology 32:261–264.

Mathiessen T, Linde A, Olding-Stenkvist BW, Wahren B (1989): Antiviral IgM and IgG subclasses in varicella-zoster associated neurological syndromes. Journal of Neurology Neurosurgery and Psychiatry 52:578–582.

Mayo DR, Boos J (1989): Varicella zoster associated neurological disease without skin lesions. Archives of Neurology 46:313–315.

Mertens G, Ieven M, Ursi D, Pattyn SR, Martin JJ, Parizel PM (1993): Detection of herpes simplex virus in the cerebrospinal fluid of patients with encephalitis using the polymerase chain reaction. Journal of the Neurological Sciences 118:213–216.

Nahass GT, Mandel MJ, Cook S, Fan W, Leonardi CL (1995): Detection of herpes simplex and varicella zoster infection from cutaneous lesions in different clinical stages with the polymerase chain reaction. Journal of the American Academy of Dermatology 32:730–733.

Pohl-Koppe A, Dahm C, Elgas M, Kühn JE, Braun RW, Meulen VT (1992): The diagnostic significance of the polymerase chain reaction and isoelectric focusing in herpes simplex virus encephalitis. Journal of Medical Virology 36:147–154.

Puchhammer-Stöckl E, Popov-Kraupp T, Heinz HX, Mandl CW, Kunz C (1991): Detection of varicella-zoster virus DNA by polymerase chain reaction in the cerebrospinal fluid from patients suffering from neurological complications associated with chickenpox or herpes zoster. Journal of Clinical Microbiology 29:1513–1516.

Quinn JP, McGeoch DJ (1985): DNA sequence of the region in the genome of herpes simplex type 1 containing the genes for DNA polymerase and their major DNA binding protein. Nucleic Acids Research 13:8143–8163.

Roberg M, Forsberg P, Tegnell A, Ekerfeldt K (1995): Intrathecal production of specific IgA antibodies in central nervous system infections. Journal of Neurology 242:390–397.

Schmidt NJ, Dennis J, Lennette EH (1977): Complement-fixing reactivity of varicella-zoster virus subunit antigens with sera from homotypic infections and heterotypic herpes simplex virus infections. Infection and Immunology 15:850–854.

Shoji H, Honda Y, Murai I, Sato Y, Oizumi K, Hondo R (1992): Detection of varicella-zoster virus DNA by polymerase chain reaction in cerebrospinal fluid of patients with herpes zoster meningitis. Journal of Neurology 239:69–70.

Troendle-Atkins J, Demmler GJ, Buffone GJ (1993): Rapid diagnosis of herpes simplex virus encephalitis by using the polymerase chain reaction. Journal of Pediatrics 123:376–380.

Tsurumi TT, Maeno K, Nishiyama Y (1987): Nucleotide sequence of the DNA polymerase gene of herpes simplex virus type 2 and comparison with the type 1 counterpart. Gene 52:129–137.

Uren EC, Johnson PDR, Montanaro J, Gilbert G (1993): Herpes simplex virus encephalitis in pediatrics: Diagnosis by detection of antibodies and DNA in cerebrospinal fluid. The Pediatric Infectious Disease Journal 12:1001–1016.

Yoshida M, Yamagami N, Tezuka T, Hondo R (1992): Case report: Detection of varicella-zoster virus DNA in maternal breast milk. Journal of Medical Virology 38:108–110.